Feb. 6, 2007

To: USPTO
Fr: John Paul Maye "Inventor"
Re: Prior Art Information Enclosed for Patent Application No. 09/520,004

Dear Sir or Madam:

Although I am no longer employed by John I. Haas, Inc., the company that owns the rights to the above patent applications, I thought I would forward you prior art information which may affect the outcome of these patent applications.

I first came up with the idea of using isomerized hop extracts (also known as aqueous alkaline solutions of isomerized hop acids which include isoalpha acids, rho-isoalpha acids, tetrahydroisoalpha acids and hexahydro-isoalpha acids) to control bacteria infections at distilleries in the summer of 1997. I was having a discussion with a distiller from Jim Beam who told me they use hops to control bacteria contamination during fermentation. Essentially they boil the hops in water to isomerize the alpha acid into isoalpha acid and then pour this mixture into the fermenter or yeast propagation tank. My thinking was a preisomerized aqueous alkaline solution of hop extract would be easier to use. We discussed the idea for performing some testing in the fall of 1997 and I even contacted some other ethanol companies like ADM and Cargill but nothing ever came of it.

As you can see from the attached articles, hops and I've later learned, hop extract, are commonly used in the manufacture of spirits. Maker's Mark, Bookers Bourbon, Jim Beam, and Charbay are just a few spirit plants that commonly use hops as a natural antimicrobial to inhibit bacteria growth during fermentation. The Maker's Mark article also mentions using of boiling water and hops and adding this mixture to yeast when propagating it. The article also states that the hops are used for their antimicrobial properties. Although some of these articles are recent they describe the processes that have been used at these plants for over 50 years.

It is commonly practiced in the fuel ethanol industry to add an antimicrobial to the yeast propagator. The antimicrobials used are generally antibiotics such as penicillin, virginiamycin, monensin or mixtures of these. Chemicals are also used like sodium metabisulfite and sodium fluoride but not widely.

The fuel ethanol process follows exactly the same process as corn whiskey. The only differences between the two are their size, and the fact that fuel ethanol plants don't need to use food grade reagents such as enzymes, nutrients and food grade antimicrobials. Some fuel ethanol plants do use food grade reagents if they also produce food grade ethanol.

Attached is a sales sheet from Aventine Renewable Energy, Inc., located in Pekin, IL. This sales sheet describes their continuous beer fermentation process and how they produce 2 million gallons of beer per day. This fuel ethanol plant claims to be a brewery.

Attached is an alcohol sales sheet from MGP Ingredients which states they are a fuel ethanol plant and a producer of food grade alcohol. They sell their food grade alcohol to many spirit manufacturers including whiskey plants. ADM, Grain Processing Corp., AE Staley, and Cargill also sell some of their ethanol to whiskey manufacturers as well as make fuel ethanol.

When it comes to beer, whisky and fuel ethanol the three processes are very similar and the last two essentially the same.

I've included two publications written by W. J. Simpson.

Simpson's 1987 paper discusses the use of hop acids (he calls resins) during the acid washing of yeast to reduce the bacteria count. He also recommends the use of hop extracts (or aqueous alkaline solutions of isomerized hop extract) during the acid washing of yeast. The ISOHOPCO2N hop product that he uses in Experimental (b) is a commercially available 30% aqueous alkaline solution of isoalpha acids. Therefore, my idea of replacing traditional hops with isomerized hop extract was not novel. The 60° EBCBU value he uses in his examples refers to 60 part per million (ppm) of isoalpha acids (also known as isohumulone). EBC = European Brewing Congress and BU = Bitterness Units. 1 BU = 1 ppm of isoalpha acids. My personal experience is that many breweries commonly add aqueous alkaline solutions of hop extract to their yeast slurry tank to reduce the bacteria load in yeast. Breweries typically use hopped wort when propagating yeast and generally don't have to add hop acids to a propagation tank like spirit plants. Because spirits plants don't perform a kettle boil like breweries they generally boil hops in some water (to produce isoalpha acids) and they add this mixture to the yeast propagation tank or fermenter.

Simpson's 1993 paper discusses the mechanism and the minimum inhibitory concentration (MIC) of isoalpha acids (also known as isohumulone) alpha acids (humulone) and beta acids (lupulone). He states that the MIC for isohumulone (isoalpha acids) is 10-15 uM which is in line with what we saw, 4 to 6 ppm. Interestingly in the early 1990's as more and more breweries started using post-fermentation hop products (isomerized aqueous alkaline solutions of hop extracts) we started seeing some breweries experience bacteria problems during fermentation. By the mid-1990's we were regularly telling breweries that use post fermentation hop products to add some aqueous alkaline isomerized hop extract to the brew kettle to inhibit bacteria growth during fermentation. We recommended that the brewer add at least 6-8 ppm of isoalpha acid or 2-4 ppm tetrahydro-isoalpha acid or 2-4 ppm hexahydro-isoalpha acid or 8-12 ppm rho-isoalpha acid.

The attached poster by Lesley Buggey can also be found on the internet via this link: www.lfl.bayern.de/ipz/**hop**fen/10585/poster1_01.pdf This poster was presented at the 48th IHGC Scientific Commission, held in Canterbury, UK August 6, 2001. This poster does a very nice job showing the MIC's for all the isomerized hop acids commercially used in brewing today and correctly stating what we saw that the more non-polar the hop acid the more antimicrobial.

I hope the attached prior art and the above information helps you with your examination of this application.

Sincerely,

John Paul Maye
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Great Falls, VA 22066
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Price $54.99 Order

About Booker Noe

Booker's ancestor, Jacob Beam, came to Kentucky in 1788. He made and sold his first whiskey in 1795. Dozens of Jacob's descendants followed his lead, including a great grandson who became the most famous Beam of all, Jim Beam.

Booker Noe, Jim Beam's grandson, is justifiably proud of his family's heritage. Part of that heritage is the Beam yeast. "We are still using the same strain of yeast that my grandfather picked up here," explains Booker. "It's a spontaneous yeast, where you make a sweet slurry of barley malt, hops, and pick your yeast up naturally out of the air. It is more than 60 years old now. My grandmother told me, 'Jim stunk us out of the house. That yeast smelled up the whole house when he was fooling with it here right after Prohibition.'"

Developing a yeast strain the old-fashioned way takes special skill. "You don't always have success when you pick this yeast up," says Booker. "You set the sweet slurry outside in the summertime or spring and let it naturally pick up yeast from the air. Lots of times, you don't have a success at getting what you are looking for and it sours out. When it ferments, you keep fermenting it from one batch to another. All of a sudden, there it is." The use of wild yeast, as opposed to a pure culture, is one of the things that makes Jim Beam Bourbon unique. "We are practical distillers," says Booker, "not scientific. It's a natural thing."

Booker is also proud of his grandfather's other accomplishments. "My grandfather was 55 years old when Prohibition came in," he says, "and it was repealed when he was 70. He still had enough spunk at 70 years old -- and that's one of the things I marvel at -- at 70 years old he came in here and built the distillery, built his warehouses, set his stills up and got that thing running and ran it for 10 years himself, until he was 80 years old, he and his son, Jeremiah. That is why Jim Beam (the company) came back after Prohibition, because the man had the know-how to do it, where a lot of them faded out during Prohibition. It is a tribute to him, a great thing."

CHARBAY Whiskey - 750ml

SKUWHISKEY

SOLD OUT*
The Story about **Double Barrel - Release One**

I grew up around our copper Alambic Pot Still in Northern California, learning to distill many different spirits from my Father, the 12th generation Master Distiller in our family.

PRINT THIS PAGE
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Double-distilling two row malted barley is a dream come true for me as a 13th generation distiller. With the addition of aromatic hops and the rich spice of new American White Oak barrels, this Hop Flavored Whiskey celebrates the ongoing creativity of our family's long distilling heritage.

Being full barrel strength, uncut and not filtered, CHARBAY Hop Flavored Whiskey is best served on the rocks. October 2002
-Marko Karakasevic

"Charbay Whiskey is a realm unto its own," says Marie Maher, General Manager of Nectar at Bellagio Resort in Las Vegas. " Think of a delicious, floral single malt. Now think of a sublime, toasty bourbon. Somewhere between these two worlds there is Charbay, a California Whiskey. This is a whiskey with an herbacious first note followed by a soft, warm caramel and lingering subtle success," says Maher.

- Distilled from Two-Row European Barley
- Grown and malted in British Columbia
- Clean heat source to emphasize natural grain flavors
- Choice aromatic hops from around the world
- Double-distilled in an Alambic Charentais Pot Still
- 7-fraction distilling technique used for purity, smoothness
- Aged in custom-made new America White Oak-barrels
- Hand painted and individually-numbered bottle
- 70 cases/ 840 bottles 750ml
- Not available for online sale to California Residents

SOLD OUT

Your Price $325.00

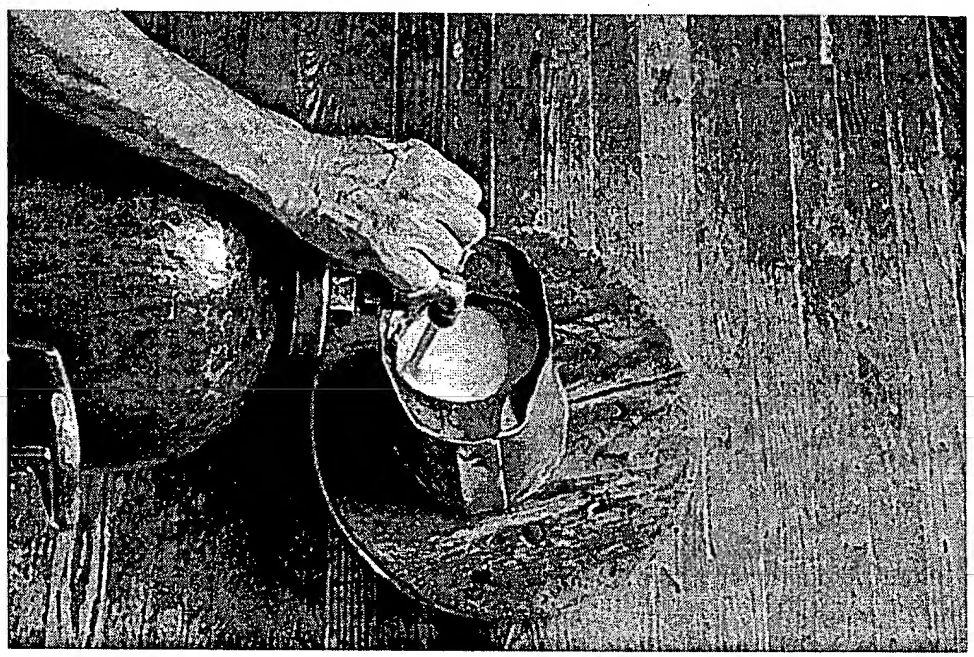
Quantity 1

ADD TO CART

Yeast Propagation

The Samuels family yeast has been preserved in a refrigerator since before Maker's Mark existed, and it is re-propagated about once a month. For each batch of bourbon, yeast is combined with boiled water and hops—used for their anti-bacterial properties.

Reader review
More Slide Shows



The McGraw-Hill Companies
BusinessWeek.com

AFTER WORK October 26, 2006, 12:10AM EST

Making a Market for Maker's Mark

Can the Loretto, Ky.-based bourbon maker hold onto its quality-intensive formula in the face of rapid growth?

by Douglas MacMillan

"If it ain't broke, don't fix it," is an appropriate characterization of the Maker's Mark approach to whiskey making, a distilling process that has remained largely unchanged since Bill Samuels, Sr. founded the company in 1952. Their time-tested recipe for high quality bourbon is followed to a T each day in the original Loretto, Ky., distillery, and millions of loyal customers the world over provide overwhelming evidence that nothing needs fixing.

The bourbon business has boomed in recent years. In 2005, more than 14 million nine-liter cases of bourbon were sold in the U.S., generating more than $1.5 billion in revenue for distillers, according to the Distilled Spirits Council of the U.S. Since 2002, the market for high-end premium and super premium bourbon has grown 6.9% and 11.9%, respectively, while demand in the least expensive category fell 3.8%.

But the proliferation of these customers—helping Maker's post double-digit growth over several consecutive years—has put the company in a pickle. How do you embrace a massive new market without sacrificing the traditional methods and high quality standards that made your brand famous to begin with?

MUSEUM QUALITY

To visit the Maker's Mark distillery, a National Historic Landmark since 1980, is to go back in time to an era when barrels were handmade by coopers, when whiskey fermented in huge wooden vats and distilled in giant copper columns, and when a tasting panel sipped, smelled, poked, and prodded each batch that came down the line.

Indeed, many of the machines used to produce Maker's Mark bourbon today are either 100-year-old antiques or custom recreations of retired equipment. Rather than modern hammer mills, Maker's Mark uses roller mills which are less efficient but have much less chance of scorching the grain and thereby creating a bitter taste.

Using wheat in the Maker's recipe forces them to use slower, open cooking mash tubs rather than pressure cookers. Neighboring bourbon distilleries such as Jim Beam and Wild Turkey abandoned such practices long ago in favor of cheaper, more efficient production. But Maker's Mark has remained committed to the consistency of its formula.

TRIED AND TRUE

Like Jim Beam, Maker's Mark is owned by Lincolnshire (Ill.)-based Fortune Brands (FO), which acquired it from distillery giant British Allied Domecq in 2005 (as well as Courvoisier cognac, Sauza tequila, Canadian Club whisky, Laphroaig single-malt Scotch, and Clos du Bois wines). Wild Turkey is part of French rival Pernod Ricard, which now owns Allied-Domecq.

Expansion of production, therefore, is a convoluted task. "Most of the time, when people think about expansion, they go to the textbooks—bigger, better, faster, more modern," says Dave Pickerell, master distiller and vice-president of operations. "We go to the archives. We pull out the drawings for the still set, go to [a custom maker], and say, 'Build us another one.' And we go back to Cleveland Welding and say, 'Build us another mash tub.' We're not trying to be technologically innovative. We're trying to do the

The McGraw-Hill Companies
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AFTER WORK October 26, 2006, 12.10AM EST

Making a Market for Maker's Mark

As any growing business knows, more revenues will also require more employees—a measure which would detract from the company's family-like group of 75 employees, many of whom have worked in the distillery at Star Hill Farm for a good chunk of their lives.

A GROWING AUDIENCE

Maker's Mark is at a major disadvantage to most other surging companies because each batch of bourbon takes about six years to produce, including the tedious aging process. This means they have to anticipate the amount of demand they will experience six years down the road.

Pickerell explains that a mathematical projection helps them make a good estimate of future demand. "We run the 'Oh no version' of a reality where everything goes wrong, then the 'Oh my' version where everything goes right. From those, we try to chart a course right down the middle." He says Maker's Mark's current middle course sees them growing by about 50% before 2012.

A blanket solution to most of Maker's Marks growing pains would be to raise their price. Currently, a one-liter bottle costs around $30 to $35, which is competitive with other small-batch bourbons such as Woodford Reserve, Basil Hayden's, and Knob Creek. Maker's Mark President and CEO Bill Samuels, Jr., son of the company's founder, refuses to budge on this matter.

FAMILY FRIENDLY FEELING

"The way we consider our market is customers, not competition," he says. "We have never thought about competition. We build relationships with customers one at a time." Even a slight rise in price, he has repeatedly declared, would undoubtedly sever the trust Samuels and his father worked to build with their customers over several decades.

Following the Fortune Brands acquisition, there was some concern that since Maker's Mark, Jim Beam, and Knob Creek—three of the leading brands of small-batch bourbon—would now be under the same umbrella, competition and quality would be threatened.

But this is only a boon to business and a fitting alliance, as far as Bill Samuels, Jr. is concerned. "The Beams and the Samuels were neighbors for more than 100 years. I grew up next door to Jim Beam and his son Jay. We're great pals, and we've never competed with each other," he says.

MacMillan is a reporter at BusinessWeek.com in New York.

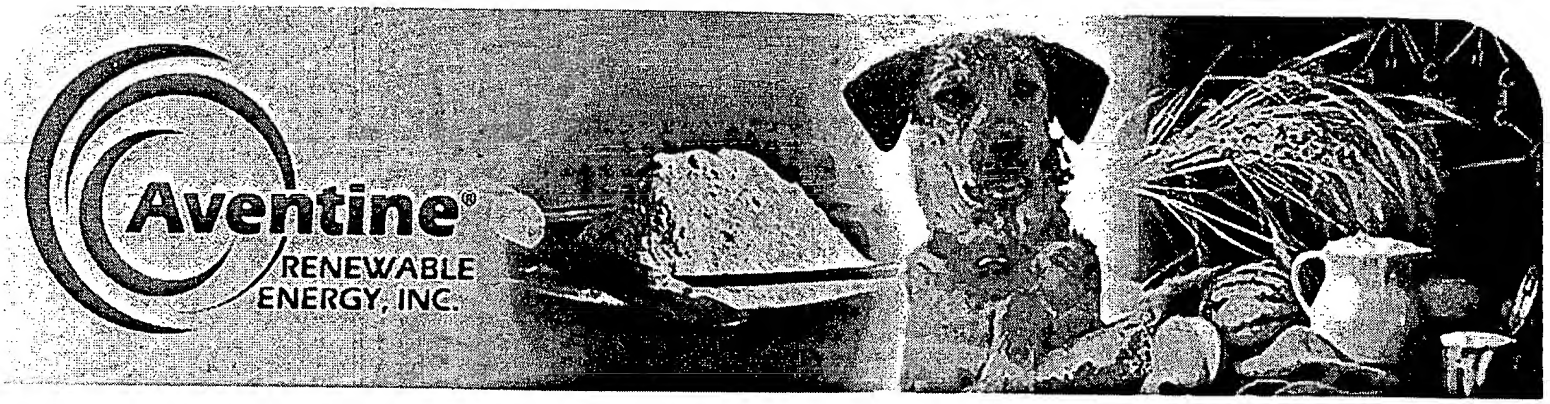
same thing over and over again."

Maker's Mark did exactly that in 2000, when it built a complete duplicate of its original distillery. This action doubled production capacity and encouraged the company to grow even faster. Six years later, demand tempts them to expand facilities further.

WHAT'S IN THE WATER?

Access to natural resources also imposes limitations. A select group of local farmers is responsible for growing all of the corn, wheat, and barley which has to meet the company's exacting standards. If they can't meet growing needs, Maker's Mark can't grow.

In addition, all of the water which goes into the whiskey comes from a limestone spring-fed lake on the distillery's premises, and the supply is certainly finite. Master distiller Pickerell claims that most loyal customers would notice a slight difference should any other water be used, a risk he's not eager to take.



Bio-Products

In 1998, Aventine Renewable Energy, Inc. formed its Bio-Products division to produce and market dried brewers yeast and other value-added products. Our mission is to provide our customers a quality product at an economic cost while providing our employees a rewarding career and our investors a profitable and satisfactory return for their capital employed.

Aventine Renewable Energy, Inc. operates 365 days a year to extract value-added products from Aventine's corn wet milling and fermentation process. Our brewers yeasts are Kosher and Chametz-free certified.

Learn more about our:

- Food grade yeast
- Feed grade yeast
- Fermentation grade yeast

Aventine Renewable Energy, Inc. operates a continuous beer fermentation process, producing more than two million gallons of beer per day. This process uses a corn starch media and brewers yeast of the saccharomyces cerviseae classification. Our plant microbiologists closely monitor the fermentation process to ensure clean, healthy fermentation without the use of antibiotics. The beer and brewers yeast stream is transported via pipeline directly to Aventine's brewers yeast plant. The brewers yeast is removed from the beer stream and immediately processed into a growing variety of products for use in animal, pet and human food, and fermentation applications.

How Bio-Products are made:

Aventine Bio-Products operates to extract value added products from Aventine's corn wet milling and fermentation process.

Click below to see how the bio-products production process works.

Bio-Products Production Flowchart (PDF, 438 KB)

The Aventine Renewable Energy, Inc. Advantages

- New, world-scale brewers yeast plant
- State-of-the-art food grade plant design
- AIB inspected facility - superior rating
- Kosher and Chametz-free certified
- Consistent supply and quality
  - No more seasonality of brewers yeast supply
  - Continuous beer fermentation process
  - No antibiotics

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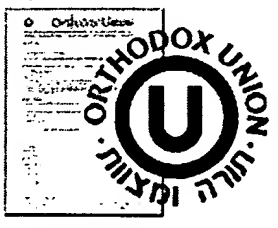

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ALCOHOL SOLUTIONS

As an important component of our integrated production process, MGPI's distillery operations supply customer needs across a diverse range of applications. We produce food grade alcohol for both beverage and industrial applications, as well as fuel grade alcohol, commonly known as ethanol.

Our high purity beverage alcohol is the source of some of the finest vodka, gin and cordials in the world. These superb products benefit from our state-of-the-art technologies and include many proprietary formulas tailored to our customers' exact specifications. Industrial applications for our food grade alcohol include food dyes, vinegar and mouthwashes, as well as cosmetics and pharmaceutical products.

INGREDIENT APPLICATIONS AND FUNCTIONS
CUSTOMER SERVICE

SYNERGISM BETWEEN HOP RESINS AND PHOSPHORIC ACID AND ITS RELEVANCE TO THE ACID WASHING OF YEAST

By W. J. Simpson

(Tennent Caledonian Breweries Limited, Glasgow)*

Received 23 January 1987

Hop resins present in brewery yeast slurries have a bactericidal action on lactic acid bacteria during the acid washing process.

Key words: *Contamination, lactic acid bacteria, yeast, hops.*

Introduction

Acid washes are commonly used to remove contaminant bacteria from pitching yeast slurries. Dean[2] suggested that acid washing cannot eliminate lactobacilli from yeast slurries and Brenner[1] found slight resistance to acidified ammonium persulphate among the lactobacilli. Van Engel[15] demonstrated that a strain of *Pediococcus cerevisiae* present in his brewery was resistant to phosphoric acid (pH 2·50) and ammonium persulphate (0·75% w/v). Recent studies on acid washing of yeast slurries[13] failed to confirm these results.

During fermentation yeast adsorbs substantial quantities of hop resins onto its surface.[3,4,7] Some brewers wash their yeast with 2–3 volumes of cold water before passing the yeast over a vibrating screen to remove these compounds.[11] This practice is considered unnecessary by ourselves and others[9] therefore the yeast used in our previous studies[13] was taken directly from the fermenter. The yeast slurry therefore contained hop resins. The possibility that hop resins present in the yeast slurry may act synergistically with acid to facilitate cell death was, therefore, tested in this work.

*Present address: Brewing Research Foundation, Lyttel Hall, Nutfield, Redhill, Surrey RH1 4HY.

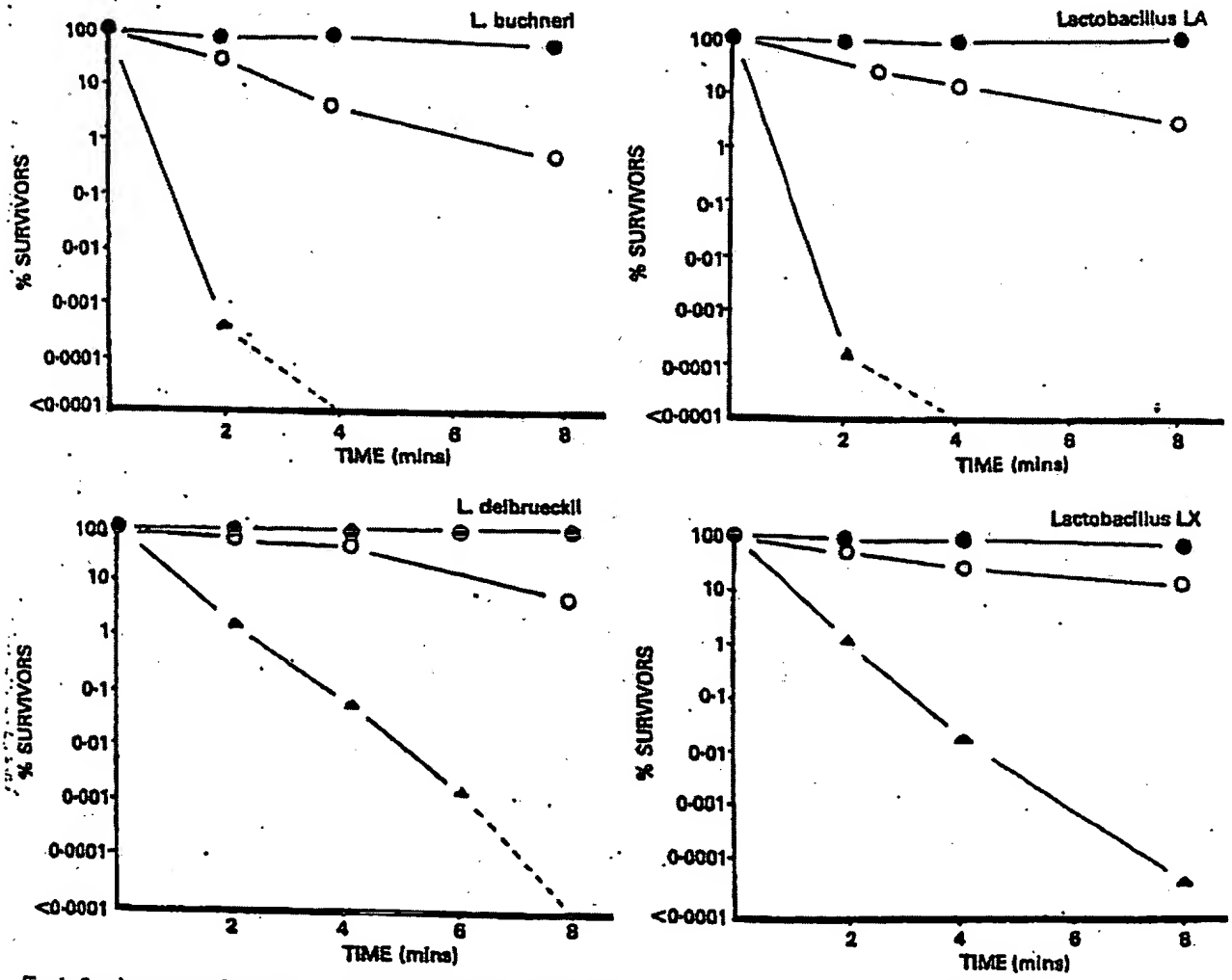

Fig. 1. Survivor curves for strains of Lactobacilli washed in phosphoric acid, pH 2·10 (○): hop resins, 60° EBCBU (●): phosphoric acid, pH 2·10/hop resins, 60° EBCBU (▲).

Results and Discussion

Hop resins are generally considered to be bacteriostatic to lactic acid bacteria.[6,8,12] Additionally it has been shown that hop resins are bactericidal to *Bacillus subtilis* 168[14] and several lactobacilli (results not presented).

As the inhibitory effects of hop resins are dependent on pH,[5,16] the effectiveness of these substances being markedly greater at lower pH, it seemed reasonable to expect synergism between hop resins and acid during washing. Figure 1 shows that synergism does occur.

Lactobacilli freshly isolated from the brewery normally possess some degree of resistance to hop resins[10] which is lost on sub-culture. Two freshly isolated strains from this brewery (LA, LX) although possessing above average resistance to the bacteriostatic effect of hop resins at pH 5·4 had little resistance to the bactericidal effect of hop resins at pH 2·10. At pH 2·10 hop resins will be lethal to lactobacilli provided they are available to the organism in sufficient quantity. Figure 1 shows the organisms tested vary in their resistance to acid which may explain the reduced effectiveness of acid washing observed by some workers when working with debittering yeast slurries.

Significance of the effect and further work

Removal of hop resins before acid treatment of yeast may reduce the lethality of the process for lactic acid bacteria and this may be of significance to breweries employing yeast de-bittering procedures or using pre-isomerised hop extracts as 100% replacement for copper hops. Conversely, the addition of hop extracts to yeast slurries during acid washing may prove useful in the occasional instance where acid resistant strains of lactobacilli are encountered. The efficiency of acid washes in the elimination of lactic acid bacteria from yeast may be influenced by the ability of the yeast to adsorb hop resins during fermentation.

The possibility that other bactericidal factors exist in brewers yeast slurries awaits investigation. Additionally, testing a number of strains of lactobacilli freshly isolated from other brewery yeasts would establish the significance of the synergistic effect described here.

Experimental

The bacteria used and conditions of incubation for the preparation of the inocula are shown in Table 1. After growth for the period stated 10 ml of each culture was washed twice in 10 ml saline (0·85 w/v NaCl). The organisms were then suspended at a level of 10^6 ml^{-1} in 200 ml of either (a) an aqueous solution of H_3PO_4 (0·5% v/v : pH 2·10): (b) an aqueous solution of isomerised hop extract (ISOHOPCO$_2$N, Pauls Hop Products, Reigate, England) hopped to a level of 60° EBCBU: (c) an acidified solution of hop extract (0·5% v/v H_3PO_4 pH 2·10: 60° EBCBU). All solutions contained 0·85% NaCl to maintain tonicity. The solutions were maintained at 5 °C (±1 °C) throughout the experiment.

TABLE 1. Organisms and incubation conditions used for the preparation of test inocula

Organism	Source	Incubation conditions	
		Temperature	Time
Lactobacillus delbrueckii	NCIB 8130	37 °C	2 days
Lactobacillus buchneri	NCIB 8516	27 °C	5 days
Lactobacillus sp. (LA)	ex plant	27 °C	5 days
Lactobacillus sp. (LX)	ex plant	27 °C	5 days

All organisms were grown in Raka-Ray medium (Difco, Centra Avenue, Surrey. Product No. 1867–17). Both brewery Lactobacilli were Gram + ve, catalase − ve, acid producing, long rods, which grew both aerobically and anaerobically.

Samples were taken at intervals during the washing period and transferred to phosphate buffered saline (pH 5·6) to arrest the washing process. Serial dilutions were made and aliquots plated in triplicate for each dilution on Raka-Ray agar. This medium was supplemented with 1g Litre^{-1} phosphatidylcholine (Sigma, USA. Product No P–5394) to neutralise hop resins carried over with the inoculum. Plates were incubated anaerobically at the temperatures shown in Table 1 for five days.

Acknowledgements:—I would like to thank Mr R. B. Miller (Senior Microbiologist, Tennent Caledonian Breweries Limited), for his helpful advice and discussion during the preparation of this paper and the Directors of Bass P.L.C. for their kind permission to publish.

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CAMBRIDGE PRIZE LECTURE. STUDIES ON THE SENSITIVITY OF LACTIC ACID BACTERIA TO HOP BITTER ACIDS

By W. J. Simpson

(BRF International, Nutfield, Surrey RH1 4HY, Great Britain)

Hop bitter acids act as mobile-carrier ionophores. They inhibit the growth of beer-spoilage bacteria by dissipating the transmembrane pH gradient. Their activity is pH dependent. Low pH favours antibacterial activity but high pH reduces it. Resistance to hop bitter acids is a stable character, associated only with beer-spoilage lactic acid bacteria. Hop-resistant organisms can maintain a larger transmembrane pH gradient and ATP pool than can hop-sensitive organisms. Prior exposure of bacteria to *trans*-isohumulone does not influence the degree of resistance to hop bitter acids. However, in some strains, exposure to *trans*-isohumulone does induce the ability to spoil beer. The chemistry of these compounds is more complex than previously thought. In aqueous solutions, such as beer, hop acids bind to metal ions and may be covalently hydrated.

Key Words: *α-acid, β-acid, bacteria, beer, hop, hop resin, iso-α-acid, spoilage*

Introduction

Compounds derived from hops have several effects. They impart a desirable bitter flavour to beer [36] and protect the beer against spoilage by lactic acid bacteria (*Lactobacillus* spp. and *Pediococcus* spp.)[10,11,14,20,24]. They enhance the stability of beer foams [3] and promote foam cling[2]. However, they can also have undesirable effects. For example, they can cause gushing of beer[13,15] and promote beer staling[9]. The iso-α-acids, the major bitter acids in beer, are responsible for the development of light-struck flavour—an off-character that develops in beer exposed to light[18].

In the last 30 years, it has been questioned whether the antibacterial properties of hop bitter acids are truly beneficial to beer stability [8,10]. Some reports have indicated that the concentration of hop bitter acids in beer may be insufficient to inhibit bacterial growth[34]. Others have suggested that beer-spoilage bacteria become acclimatized to hop bitter acids[17], leading to a further diminution in the protective effect of the compounds. Also, the widespread use of pasteurization has reduced the reliance on the natural defenses of the beer against bacterial spoilage.

Prior to the present study, the mechanism by which hop bitter acids inhibit growth of beer-spoilage bacteria had not been established. In a meticulous study, Teuber and co-workers showed that hop compounds and their derivatives interfere with the function of the cell plasma membrane in the aerobic organism *Bacillus subtilis*[20,35]. However, this organism cannot grow in beer and beer-spoilage bacteria differ from *B. subtilis* in the physiology of their cell membranes.

Several questions were unaswered. (i) Why do hop bitter acids inhibit bacterial growth? (ii) Why do some bacteria resist the antibacterial action of hop bitter acids? (iii) Do some bacteria spontaneously develop resistance to hop bitter acids? and (iv) To what extent do hop bitter acids protect beer form bacterial spoilage? The results of studies carried out in each of these areas have been summarized here.

Hop Compounds and Their Derivatives

The compounds used in these studies are shown in Figure 1. They are colupulone (a β-acid), (-)-humulone (an α-acid), *trans*-isohumulone (an iso-α-acid) and *trans*-humulinic acid (a hydrolysis product of (-)-humulone and isohumulone). They were isolated using established procedures and their identity and purity checked by analysis of melting point,

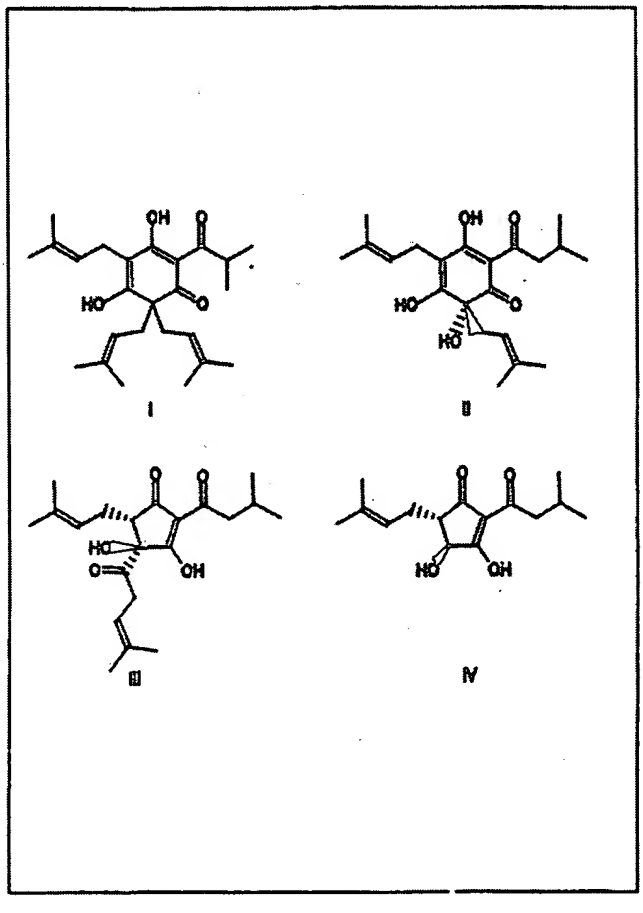

Fig. 1. Chemical structures of hop bitter acids. (I) colupulone, (II) (-)-humulone, (III) *trans*-isohumulone, (IV) *trans*-humulinic acid.

determination of ultra-violet (UV) and infra-red (IR) absorption spectra, determination of molecular weights by mass spectrometry, and the use of ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy[24].

Factors Affecting the Antibacterial Action of Hop Compounds and Their Derivatives

Measurement of the minimum inhibitory concentration

One of the best ways to assess antibacterial activity is to measure the minimum inhibitory concentration (MIC) of

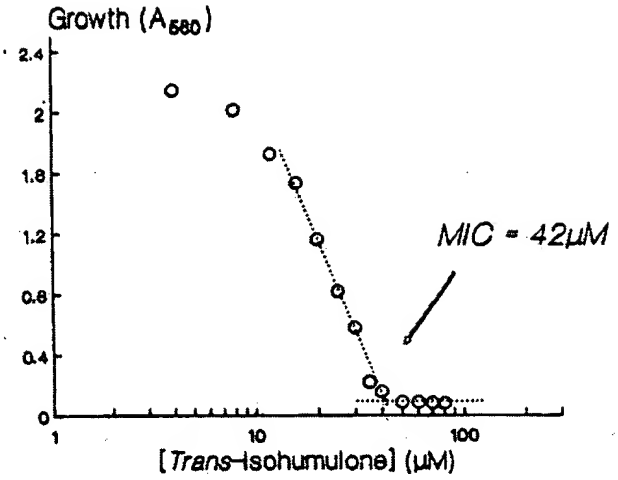

FIG. 2. Typical determination of the minimum inhibitory concentration (MIC) of a hop bitter acid. The test compound was *trans*-isohumulone and the test organism *Lactobacillus brevis* IFO 3960. The organisms were grown in modified MRS medium (pH 5.2) for 48 h.

the substance. The MIC is the smallest concentration of a substance that inhibits growth of the test organism in a given time. Several tubes of growth medium are prepared, each containing a different concentration of the hop bitter acid. Each tube is then inoculated. After incubating the cells at 25°C for 48 h, the optical density of the culture is measured to estimate the extent of growth. A typical set of results for an MIC test is shown in Figure 2.

Effect of pH on the MIC

It has been known for almost 70 years that the antibacterial activity of certain hop compounds is influenced by the pH value of the test medium[7]. However, the effect has not previously been quantified or explained. With *Lactobacillus brevis* IFO 3960, the antibacterial activity of each of the four hop bitter acids varied, sometimes markedly, over the pH range 4–7 (Figure 3a). The change in MIC with respect to the change in pH was least with colupulone, greater with (-)-humulone and most marked with *trans*-isohumulone and *trans*-humulinic acid. As the pH value changed from 4 to 7, there was an 800-fold decrease in the antibacterial activity of *trans*-hisohumulone. To explain these results we must consider the nature of hop bitter acids.

Compounds such as *trans*-isohumulone are weak acids. They ionize in aqueous solution. The degree to which they ionize is controlled by the ionization constant (Ka) of the hop bitter acid and the pH of the solution. (pKa is the negative logarithm of Ka.) A detailed study of the ionization properties of hop bitter acids showed that their ionization could not be described by single pKa values. Instead, only approximate, or more correctly, equilibrium pKa (pKa_{equil}) values were obtained (see below for a fuller discussion of this effect). With colupulone, (-)-humulone, *trans*-isohumulone and *trans*-humulinic acid, pKa_{equil} values of 6.1, 5.0, 3.1 and 2.7 respectively were obtained in aqueous solution[26].

These values could be used to provide a satisfactory explanation of the effect of pH on the inhibitory properties of hop bitter acids. If the pKa value of the test compound and the pH values of the solution are known, the concentration of the ionized and undissociated forms of the compound at each pH can be calculated[1]. When the amount of the undissociated form of each hop bitter acid required to inhibit bacterial growth at each pH value was calculated, it was found that a relatively constant amount was needed in each case (Figure 3b). This showed that undissociated forms of the bitter acids are responsible for antibacterial activity[33].

This finding has an important practical consequence: small changes in beer pH value cause large changes in the antibacterial activity of the hop bitter acids in the beer. A change in pH of as little as 0.2 can reduce the protective effect of hop compounds by as much as 50%[30].

Effect of cations on the MIC

Monovalent cations (such as K^+, Na^+ and Rb^+) and divalent cations (such as Mg^{2+}, Mn^{2+} and Ca^{2+}) affect the antibacterial activity of *trans*-isohumulone[33]. The antibacterial activity of this hop bitter acid was increased by monovalent cations but decreased by divalent cations. Figure 4 shows that the effects of the cations are dependent on their concentration. These ions did not affect the growth yield of control cell suspensions without *trans*-isohumulone. An exception, however, was Mg^{2+}, which inhibited growth of *L. brevis* IFO 3960 when it was present in the growth medium

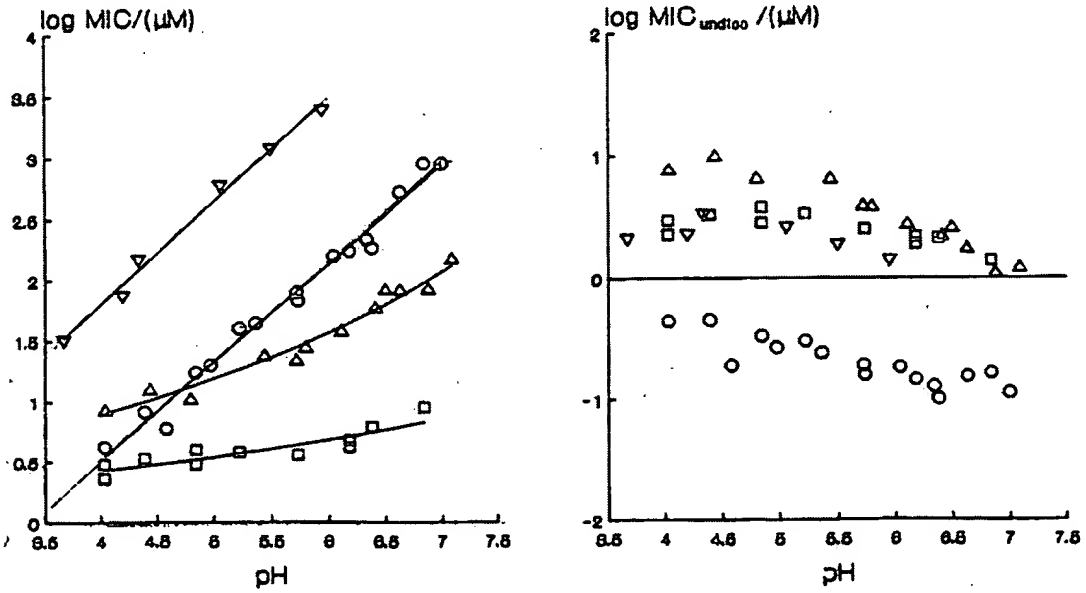

FIG. 3. Effect of pH on the antibacterial activity of hop bitter acids. (a) Relationship between pH and MIC. (b) Relationship between pH and the MIC of the undissociated from (MIC_{undiss}) of each hop acid. The test organism was *Lactobacillus brevis* IFO 3960. □, colupulone; △, humulone; ○, *trans*-isohumulone; ▽, *trans*-humulinic acid.

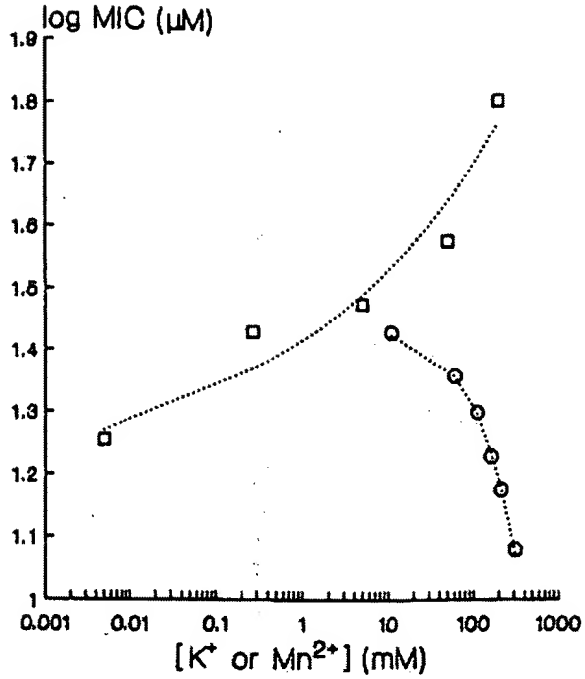

FIG. 4. Effect of the concentration of K^+ (O) and Mn^{2+} (□) on the MIC of *trans*-isohumulone for *Lactobacillus brevis* IFO 3960.

at high concentration. *Trans*-isohumulone could rescue the cells from the toxic effects of Mg^{2+}. Low concentrations of the hop bitter acid allowed normal growth to proceed in the presence of Mg^{2+}. In this situation, *trans*-isohumulone acted not as an antibacterial agent, but as a growth promoter. Higher concentrations of *trans*-isohumulone inhibited growth of the cells.

Mechanism by Which *Trans*-isohumulone Inhibits Bacterial Growth

Bacteriostatic or bactericidal?

Concentrations of *trans*-isohumulone lower than the MIC reduced the growth rate of sensitive organisms. When applied at the MIC, *trans*-isohumulone immediately arrested the growth of *L. brevis* IFO 3960 (Figure 5). The affected cells stopped growing but were not killed. If the organisms were suspended in fresh growth medium, not containing the hop acid, they grew again after a lag of about 7 h. The lag phase of untreated organisms under these conditions was 1 h. On prolonged exposure to *trans*-isohumulone, at concentrations greater than the MIC for growth, the organisms died. The rates of death induced by 2x and 3x the MIC were similar (Figure 6). Clearly, *trans*-isohumulone can have a bacteriostatic or bactericidal action depending on the conditions employed[25,30].

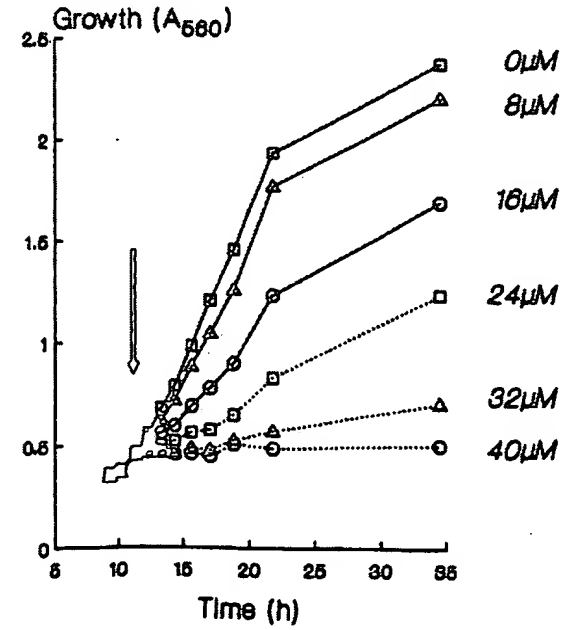

FIG. 5. Inhibition of the growth of *Lactobacillus brevis* IFO 3960 by *trans*-isohumulone. The hop bitter acid was added to the cell suspension at the stage indicated.

Metabolic effects

A detailed study of the effect of *trans*-isohumulone on the metabolic activities of *L. brevis* IFO 3960[25] showed the following. (i) *Trans*-isohumulone reduces the cellular ATP content of non growing cells metabolizing glucose (Figure 7). It can either increase or decrease the ATP content of growing cells depending on the conditions and time of contact. (ii) *Trans*-isohumulone does not cause general disruption of cell permeability. (iii) It inhibits the uptake by cells of [^{14}C]-L-leucine (Figure 8). (iv) It promotes slow efflux of [^{14}C]-L-leucine from preloaded cells. (v) It dissipates the transmembrane pH gradient (ΔpH) of cells completely but has less effect on the membrane potential (Table 1). (vi) It does not inhibit the activity of the proton-translocating ATPase.

Isohumulone acts as an ionophore

The reduction in intracellular pH caused by *trans*-isohumulone is not due to inhibition of ATP generation or to inhibition of the proton-translocating ATPase[25]. Might hop-derived compounds such as *trans*-isohumulone inhibit bacterial growth in the same way as weak acid food preservatives? Such materials dissipate ΔpH, in an electroneutral process, by accumulating in the intracellular matrix. As they dissociate into protons and anions, they reduce intracellular pH[19]. This mechanism is the basis of a widely used method for measurement of intracellular pH[5]. However, hop compounds and their derivatives inhibit growth of *L. brevis* IFO 3960 at concentrations as low as 2 μM[33]. In contrast, weak acid food preservatives, such as benzoic acid or sorbic acid, are only active at millimolar concentrations[19].

An alternative explanation is that hop-derived compounds

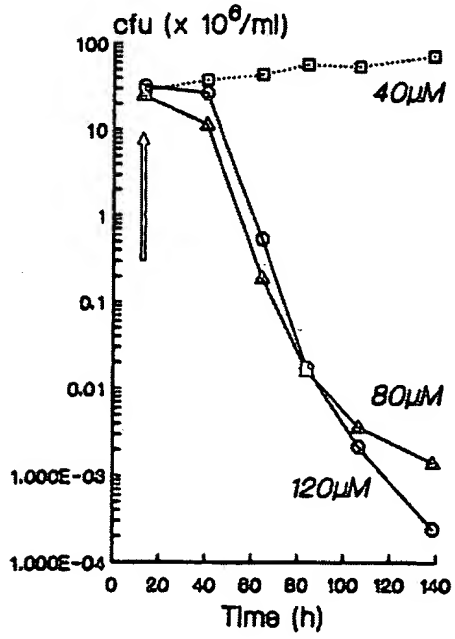

FIG. 6. Effect of *trans*-isohumulone on the viability of *Lactobacillus brevis* IFO 3960. *Trans*-isohumulone was added to the cells at the stage indicated. Prolonged exposure to the hop bitter acid at concentrations above the MIC killed the cells.

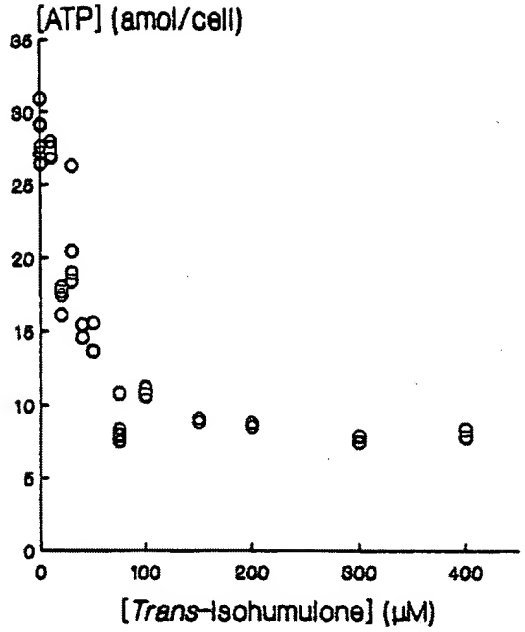

FIG. 7. Effect of *trans*-isohumulone on the ATP content of non-growing cells of *Lactobacillus brevis* IFO 3960. Orgnaisms were mixed with *trans*-isohumulone and incubated for 60 min at 25°C.

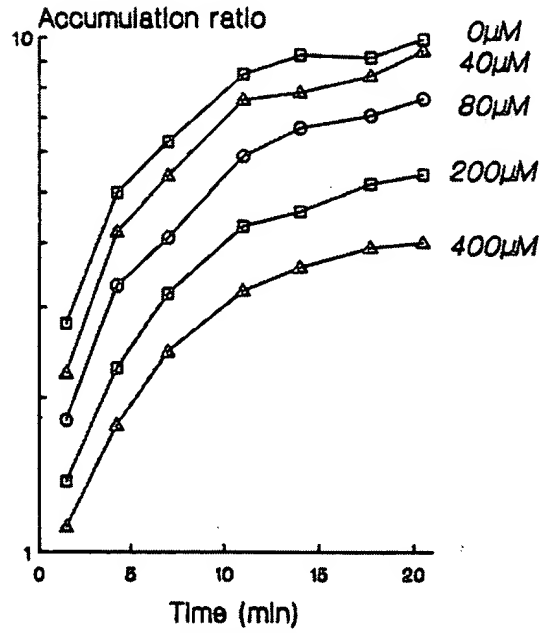

FIG. 8. Inhibition of [U-^{14}C]-L-leucine uptake by *Lactobacillus brevis* IFO 3960.

TABLE I. Effect of *trans*-isohumulone on the transmembrane pH gradient (ΔpH) membrane potential (ΔΨ) of *Lactobacillus brevis* IFO 3960

	ΔpH	ΔΨ
Control	0.52 ± 0.002	−71.5 ± 3.1 mV
trans-isohumulone (400μM)	0.09 ± 0.09	−42.7 ± 9.9 mV

ΔpH and ΔΨ were determined from the transmembrane pH gradient of [^{14}C]-salicylic acid and [^{3}H]-tetraphenylphosphonium ions respectively.

act as ionophores. Ionophores, which catalyse transport of ions across biological membranes, fall into two classes[16]. True ionophores move freely through the plasma membrane. *Quasi*-ionophores from stationary pores in the membrane. The activity of true ionophores is sensitive to changes in temperature, unlike that of *quasi*-ionophores. The action of protonophores, a group of ionophores that move protons across cell membranes, can be monitored by potentiometry.

If organisms are suspended in an unbuffered medium and the pH of the medium is lowered by addition of hydrochloric acid, an artificial pH gradient is set up. The pH value inside the cells is higher than that outside the cells. As bacterial cell membranes are impermeable to protons, it can take several hours for the intracellular and extracellular pH values to equalize. If a protonophore is added to the cell suspension, the extracellular pH value increases rapidly. The effects of *trans*-isohumulone are shown in Figure 9. Protons from the extracellular medium move across the cell membranes and into the intracellular fluid. The intracellular pH falls and the extracellular pH rises. The direction of proton movements can be reversed by adding $MnCl_2$ to the extracellular medium (Figure 10).

Also, the effects of *trans*-isohumulone are temperature-dependent. The ionophoric effects of the hop bitter acid are reduced at lower temperatures (Table II).

These results suggest that *trans*-isohumulone is a mobile-carrier of ions. At temperature above the 'melting point' of the membrane lipids, it traps protons at one membrane surface and exchanges them for Mn^{2+} (or other divalent cations) at the other. The reduction in intracellular pH that results leads to inhibition of nutrient transport and starvation of the affected organisms (Figure 11). The stoichiometry of the process is not yet known. At lower temperatures, the mobility of *trans*-isohumulone in the cell membrane is reduced, restricting the ionophoric activity of *trans*-isohumulone.

These effects can be demonstrated using genetically-modified bacteria. Organisms that have been transformed with the *lux*A/B gene from *Vibrio fischeri* catalyse the following reaction

$$FMNH_2 + O_2 + R\text{-}CHO \Rightarrow FMN + R\text{-}COOH + H_2O + \text{light}$$

(where R-CHO represents a long-chain aldehyde, such as dodecanal, R-COOH the corresponding acid, and $FMNH_2$ and FMN the reduced and oxidized forms of flavin mononucleotide respectively). The *lux*A/B gene codes for bacterial luciferase. Hop bitter acids reduce the dodecanal-dependent light emission from *lux*A/B-transformed cells of *Lactococcus lactis* susbsp. *diacetylactis* F712 since they reduce intracellular pH[28]. This finding allowed an assay for hop bitter acids (and

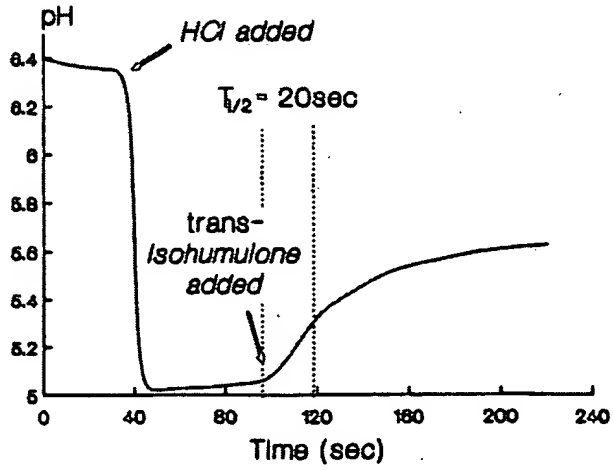

FIG. 9. Influx of protons into cells of *Lactobacillus brevis* IFO 3960 induced by 100 μM *trans*-isohumulone. The temperature of the suspension was 25°C.

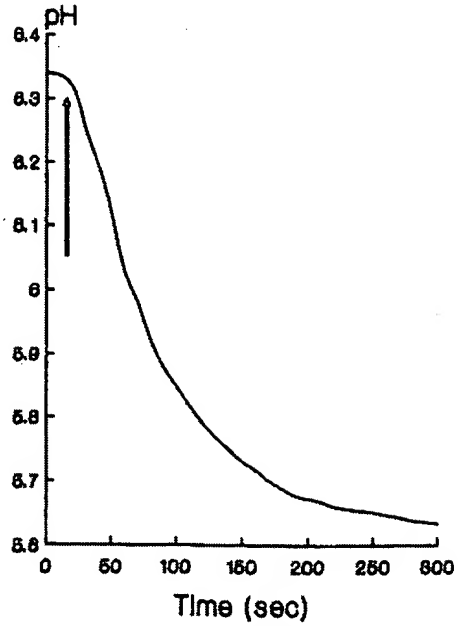

FIG. 10. Effect of Mn^{2+} on the direction of proton movements induced by *trans*-isohumulone. The hop bitter acid (100 μM) was added at the stage indicated. The test organism was *Lactobacillus brevis* IFO 3960 and the temperature of the suspension was 25°C.

TABLE II. Effect of temperature on ionophoric activity of *trans*-isohumulone against *Lactobacillus brevis* IFO 3960

Temperature (°C)	$T_{1/2}$ (min)*
0	>30
5	5.6
10	2.0
20	0.3

*$T_{1/2}$ is a measure of the cells' permeability to protons. High values show that the cells have low permeability and low values show that the permeability has increased.

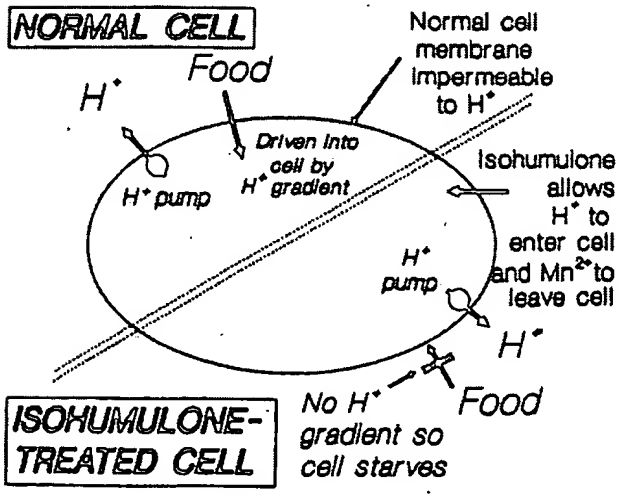

FIG. 11. Mechanism by which *trans*-isohumulone inhibits growth of bacteria.

other ionophores) to be devised (Figure 12). The effects of hop bitter acids on the intracellular pH of the organism could also be monitored using the intracellular luciferase activity as a molecular indicator of pH[27].

Incidentally, the finding that isohumulone is an ionophore allows an explanation of a previous observation that such compounds help to kill lactic acid bacteria during acid washing of yeast[22,23]. During acid washing, hop bitter acids accelerate the ingress of protons into the bacterial cells thereby enhancing the lethal effects of the acid. This phenomenon has significant consequences for brewers who employ post-fermentation bittering, but acid wash their yeast. Under conditions in which hop bitter acids are absent, the efficacy of the acid washing process against Gram-positive bacteria is reduced.

CHEMISTRY OF THE HOP BITTER ACIDS

Ability of hop bitter acids to bind metal ions

The ionophoric properties of *trans*-isohumulone clearly depend on its ability to complex metal ions. Several workers have studied this property[4] but they have not drawn attention to the interaction of *trans*-isohumulone with Mn^{2+}. In methanol, *trans*-isohumulone binds to Mn^{2+}. This changes the UV absorption spectrum of *trans*-isohumulone. The affinity of *trans*-isohumulone for other physiologically significant divalent cations (e.g. Mg^{2+}, Ca^{2+}) is less than that for Mn^{2+} (Hughes & Simpson, unpublished results).

Trans-isohumulone cannot carry protons into cells unless a monovalent cation, such as K^+, is present in the extracellular medium. This is probably the reson why the MIC of *trans*-isohumulone is affected by the K^+ concentration of the growth medium (Figure 4). Recently, we found that *trans*-isohumulone is unable to bind to K^+ unless a divalent cation is present[32]. Thus, the ability of hop bitter acids to simultaneously bind to two or more cations may be crucial to their antibacterial action.

Covalent hydration of hop bitter acids

Several methods were used to compare the ionization behaviour of colupulone, (-)-humulone, *trans*-isohumulone and *trans*-humulinic acid. Two distinct pKa values were obtained for each hop acid depending on the method used[26]. For example, with *trans*-isohumulone, conductionmetric and

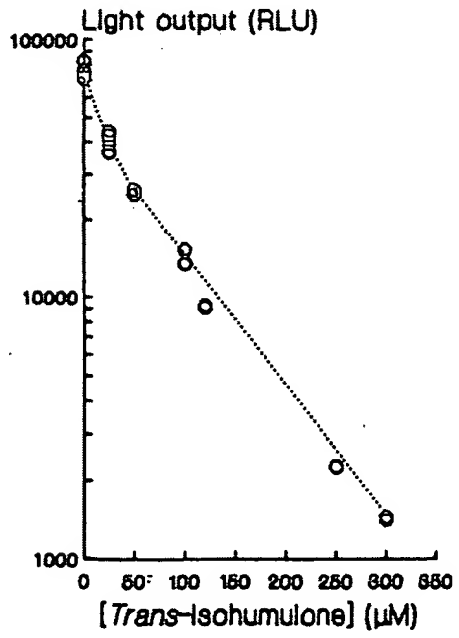

FIG. 12. Assay of the concentration of *trans*-isohumulone with *lux*A/B-transformed cells of *Lactococcus lactis* subsp. *diacetylactis* F712.

potentiometric analyses showed that the pKa was *ca* 3.1, but measurements made by spectrophotometry and by solubility determination gave a pKa value of 1.3. There are several possible explanations of this effect. The ability of the compounds to exist as ketonic or enolic tautomers could be involved. Alternatively, their ability to bind metal ions could be responsible. Control studies eliminated these possibilities. In aqueous solution, hop compounds and their derivatives, are probably covalently hydrated[26]. The hydration reaction is an equilibrium reaction (Figure 13). Without water, for example in hexane solution, the compounds dehydrate. In the presence of water, the carbonyl groups of the hop acid are hydrated. This reaction, which can be detected by spectrophotometry and by solubility determination, gives rise to "pseudo" pKa values. The significance of covalent hydration to the antibacterial activity of hop bitter acids, and to their behaviour in the brewing process, is not known at present.

Resistance of Bacteria to Hop Bitter Acids

Incidence of hop resistance

Most strains of lactic acid bacteria are inhibited by 10–15 μM *trans*-isohumulone in modified MRS medium at pH 5.2. Some *Lactobacillus* spp. and *Pediococcus* spp. can resist concentrations of greater than 80 μM (Often 120–200 μM) *trans*-isohumulone. Invariably, such hop-resistant organisms have been isolated from spoiled beer. Hop-resistant organisms have not yet been found in the genus *Leuconostoc* or *Lactococcus*[29], bacteria which have no history of beer spoilage.

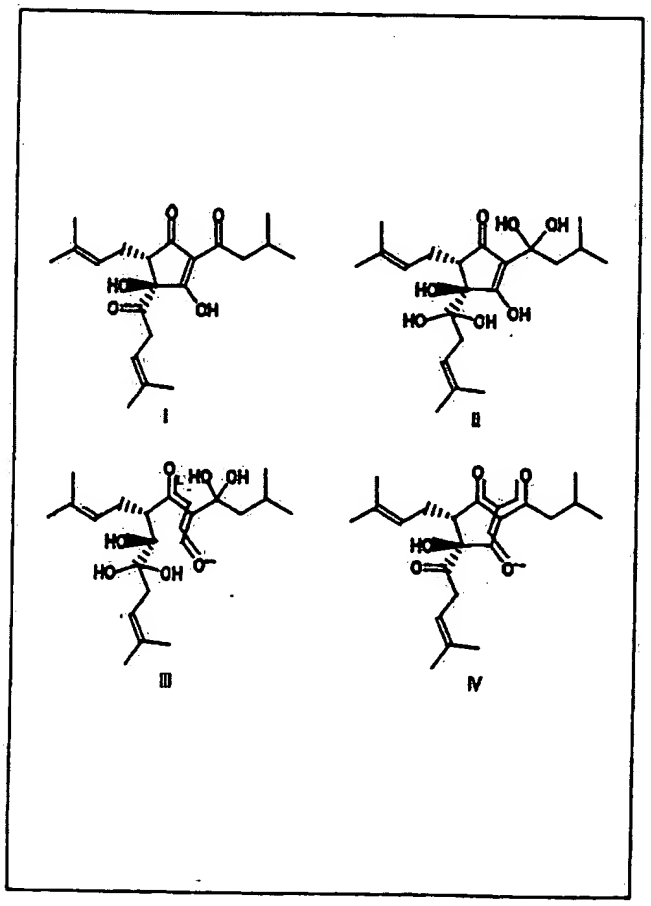

Fig. 13. Different molecular forms of *trans*-isohumulone. (I) dehydrated undissociated, (II) hydrated undissociated, (III) hydrated ionized, (IV) dehydrated ionized.

Stability of hop resistance

Richards and MacRae[17] reported that lactic acid bacteria easily develop resistance to hop compounds. They showed that when the organisms were grown in a medium which contained humulone or isohumulone, the concentration needed to inhibit their growth progressively increased. However, we have been unable to duplicate these results. Perhaps the increases in resistance were caused not by adaptation of the organisms, or by mutation, but rather by selection of hop resistant contaminants in the test culture.

Both resistance and sensitivity to *trans*-isohumulone appear to be relatively stable characters[6,29]. The degree of resistance of several lactic acid bacteria could not be altered to any great extent by growth in the presence of isohumulone for up to eight subcultures. Also, growth of the cells without isohumulone for up to ten subcultures did not change their MIC. Indeed, with *Lactobacillus brevis* BSO 310, the degree of resistance could not be altered by UV mutagensis or by plasmid curing[6]. This finding can be exploited. For example, microbiological media supplemented with hop bitter acids can be used to detect hop-resistant lactic acid bacteria in the presence of hop-sensitive organisms[30]. The growth of lactic acid bacteria that belong to genera not associated with beer spoilage can also be prevented by using vancomycin in the growth medium[31].

Features of hop resistance[6]

Resistance to *trans*-isohumulone is associated with resistance to the related hop acids (-)-humulone and colupulone. This suggests that the molecular structure of the hop acid does not markedly affect the resistance mechanism. Hop-resistant bacteria are not better able to resist other antibacterial agents or antibiotics. Elimination of all plasmid DNA from hop-resistant cells did not alter their resistance to *trans*-isohumulone. In *L. brevis* BSO 310 (and probably in lactic acid bacteria generally), hop resistance is a stable character coded for by chromosomal DNA.

The response of hop-sensitive and hop-resistant bacteria to hop compounds could not be predicted from a knowledge of cell or colony morphology, pH range for growth, carbohydrate utilization pattern, products of metabolism or expression of cellular proteins[6]. The only way to tell whether a bacterium is hop-resistant is to measure its sensitivity to hop bitter acids!

Mechanism of Resistance to Hop Bitter Acids

The mechanism by which beer-spoilage lactic acid bacteria resist *trans*-isohumulone was also investigated (Simpson & Fernandez, unpublished results). Resistance to this hop bitter acid was not due to an ability of the bacteria to convert it to a less inhibitory substance. There were no differences in the amounts of *trans*-isohumulone bound to hop-sensitive and hop-resistant organisms. Therefore, an inability of the antibacterial agent to reach its target was not responsible for hop resistance. Resistant cells could resist hop bitter acids in both simple and complex growth media. Unlike sensitive organisms, resistant organisms maintained a transmembrane pH gradient and ATP pool in the presence of *trans*-isohumulone. These results suggest that hop resistance has its origins in the cell membrane.

Relationship Between Hop Resistance and Beer-Spoilage Ability

If bacteria are isolated from spoiled beer on agar plates, then returned to fresh beer, they often fail to grow[12,17]. However, if they are first cultured several times in a mixture of liquid growth medium and beer they become "acclimatized" and able to grow in beer. The chance of success of this procedure is increased if the ratio of beer to culture medium is gradually increased over several subcultures. Some explain this phenomenon on the basis that the organisms, which are hop-resistant when growing in beer, lose their

resistance to hop bitter acids when they are grown on microbiological culture media[17]. The resistance to hop compounds can then be restored by exposing them to mixtures of media and (hopped) beer. This explanation is simple but inconsistent with the experimental data. Hop resistance is a stable character. It does not change, to any significant extent, when the growth conditions are altered[29].

However, exposure to hop bitter acids does evoke a response in hop-resistant bacteria. For example, the type and quantity of metabolic end products of glucose metabolism are altered by sub-inhibitory concentrations of *trans*-isohumulone[29]. Also, changes in the protein composition of the cells be detected using sodium dodecyl sulphate polyacrylamide gel electrophoresis (Fernandez & Simpson, unpublished results).

If induction of beer-spoilage activity by beer was not caused by an increase in hop resistance, then it might result from a phenotypic change promoted by isohumulone. To test this hypothesis we grew organisms in modified MRS medium, with and without iso-α-acids. With hop-resistant bacteria, cells that had not been exposed to iso-α-acids could not spoil beer. However, those that had been exposed to 45 μM iso-α-acids were able to grow in beer[29]. While this effect cannot yet be explained at the molecular level, it can be exploited. The brewery microbiologist can now acclimatize bacteria to growth in beer using a simple procedure. He or she can use this method to check whether an organism can grow in beer or is innocuous. Alternatively, the technique can be used in the form of a "challenge test". This allows the "spoilibility" of a beer to be assessed before if is despatched from the brewery.

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THE ANTIBACTERIAL ACTIVITY OF HOP COMPOUNDS

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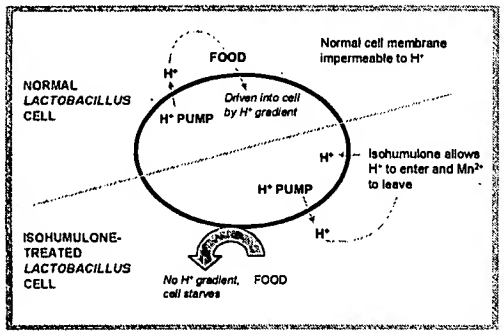

FIGURE 1. EFFECT OF ISO-ALPHA-ACIDS ON CELL MEMBRANES.

METHODS

- iso-α-acids added to modified MRS broth, pH range 3.6 – 6.4
- inoculated with beer spoilage *Lactobacillus brevis*
- incubation (in dark) at 25°C for 48 hours
- Minimum Inhibitory Concentration (MIC) of each compound determined by measuring absorbance at 560 nm
- hydrophobicity determined using reverse-phase HPLC (*Hughes* et al, 1996)

The antibacterial activities of 12 iso-α-acids were investigated:

- *cis-* & *trans-* isomers of 3 naturally occurring iso-α-acids
- 5 chemically reduced iso-α-acids
- reduced iso-α-acid mixture

i.e.

TIC	*trans*-isocohumulone
CIC	*cis*-isocohumulone
TIH	*trans*-isohumulone
TIA	*trans*-isoadhumulone
CIH	*cis*-isohumulone
CIA	*cis*-isoadhumulone
RIH	reduced iso-α-acids
DH1	dihydroiso-α-acid 1
DH2	dihydroiso-α-acid 2
TTIC	*trans*-tetrahydroisocohumulone
HIC	hexahydroisocohumulone
HIH	hexahydroisohumulone

INTRODUCTION

Hops are used to impart bitterness and aromatic flavours to beer, however certain compounds also possess antimicrobial properties. The iso-α-acids in particular possess strong antibacterial action against Gram positive bacteria (*Simpson & Smith*, 1992).

Iso-α-acids act as mobile carrier type ionophores, causing breakdown of the trans-membrane proton gradient of susceptible cells (*Simpson*, 1993). The cell is therefore unable to take up nutrients (Figure 1).

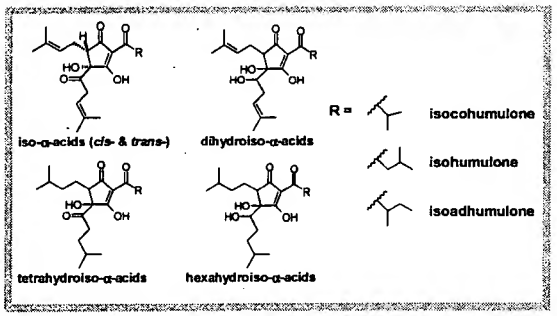

FIGURE 2. ISO-ALPHA-ACIDS STUDIED

Increased hydrophobicity leads to a greater antimicrobial activity (Figure 3). The more hydrophobic, reduced iso-α-acids are more antimicrobial than their naturally occurring analogues, and the degree of reduction is important (Figure 4). Increased hydrophobicity results in increased lipophilicity. This would render a compound more prone to interaction with the cell membrane, which could explain the observed effects.

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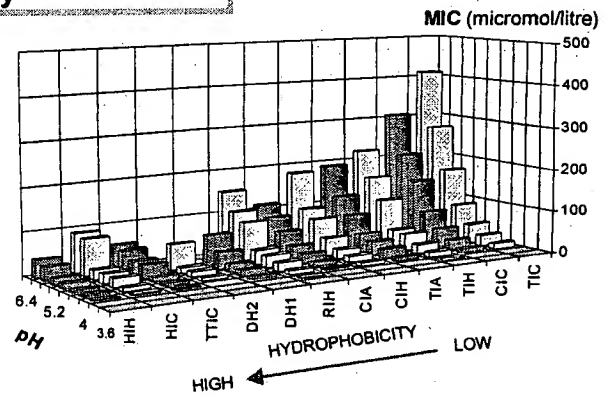

FIGURE 3. EFFECT OF pH AND HYDROPHOBICITY ON ANTIMICROBIAL ACTIVITY

(n.b. the lower the MIC value, the higher the antimicrobial activity)

Figure 3 also shows that antimicrobial activity is higher at lower pH (first noted by Shimwell, 1937). It is the undissociated forms of the iso-α-acids that possess antimicrobial properties. As they are weakly acidic, at lower pH there will be a greater concentration of the undissociated iso-α-acids.

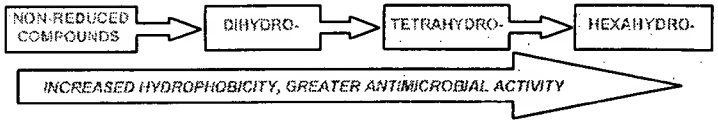

FIGURE 4. DEGREE OF HYDROPHOBICITY OF HOP COMPOUNDS.

CONCLUSIONS

- reduced hydrophobicity & lower pH ↑ increased antimicrobial activity.
- chemically reduced iso-α-acids ↑ more antimicrobial.
- reduced hop compounds often used to prevent 'lightstruck' flavour.
- for sterile filtered, unpasteurised beers that are prone to microbiological problems, the addition of reduced hop compounds ↑ additional benefit of improved microbiological stability.

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Sheila Lee for technical assistance, Louise Bolshaw for purification of hop compounds and Paul Hughes for hydrophobicity data